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Daily Rhythm in Pineal Phosphodiesterase (PDE) Activity Reflects Adrenergic/3′,5′-Cyclic Adenosine 5′-Monophosphate Induction of the PDE4B2 Variant

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The pineal gland is a photoneuroendocrine transducer that influences circadian and circannual dynamics of many physiological functions via the daily rhythm in melatonin production and release. Melatonin synthesis is stimulated at night by a photoneural system through which pineal adenylate cyclase is adrenergically activated, resulting in an elevation of cAMP. cAMP enhances melatonin synthesis through actions on several elements of the biosynthetic pathway. cAMP degradation also appears to increase at night due to an increase in phosphodiesterase (PDE) activity, which peaks in the middle of the night. Here, it was found that this nocturnal increase in PDE activity results from an increase in the abundance of PDE4B2 mRNA (~5-fold; doubling time, ~2 h). The resulting level is notably higher (>6-fold) than in all other tissues examined, none of which exhibit a robust daily rhythm. The increase in PDE4B2 mRNA is followed by increases in PDE4B2 protein and PDE4 enzyme activity. Results from *in vivo* and *in vitro* studies indicate that these changes are due to activation of adrenergic receptors and a cAMP-dependent protein kinase A mechanism. Inhibition of PDE4 activity during the late phase of adrenergic stimulation enhances cAMP and melatonin levels. The evidence that PDE4B2 plays a negative feedback role in adrenergic/cAMP signaling in the pineal gland provides the first proof that cAMP control of PDE4B2 is a physiologically relevant control mechanism in cAMP signaling. (*Endocrinology* 148: 1475–1485, 2007)

THE PINEAL GLAND functions through the hormone melatonin to influence a broad range of circadian and circannual functions. These effects are mediated by the daily rhythm in circulating melatonin, characterized by a large nocturnal increase. This provides a precise, reliable, and reproducible indication of the duration of the night period. A signal of this nature is necessary to transduce seasonal changes in day length into a useful hormonal signal that optimally coordinates environmental changes in day length with physiological changes (1).

The daily rhythm of melatonin production in mammals is controlled to a large degree by adrenergic stimulation of cAMP, which has rapid effects on the expression of a number of genes. Perhaps the best studied example is the effect of cAMP on the activity of arylalkylamine *N*-acyltransferase (AANAT) (1), the penultimate enzyme in melatonin synthesis (2, 3). Changes in AANAT activity regulate changes in melatonin production in a precise manner, earning the enzyme the label the timezyme (4).

cAMP also controls the expression of other genes involved in melatonin synthesis, including the induction of *Mat2a*, which encodes an enzyme that synthesizes *S*-adenosyl methionine, the cofactor of the last enzyme in the melatonin pathway (5). It is also likely that cAMP increases the activity of tryptophan hydroxylase, the first enzyme in melatonin synthesis (6, 7). The regulatory targets of cAMP are not limited to the essential elements of the melatonin synthetic pathway but also include *Dio2* (8, 9), *pgPept1* (10), *Fra-2* (11), *ICER* (12), *RZRβ/RORβ* (13), *NGFI-B* (14), α_{1B}*-AR* (15), and *MKP-1* (16). cAMP-induced expression of these genes appears to reflect the existence of a complex cAMP-controlled system that regulates the pineal response to adrenergic stimulation, thereby contributing to biological timing by fine-tuning the melatonin signal. The extent and functional organization of this regulatory system have not been established.

Adrenergic stimulation of pineal cAMP is controlled in a circadian manner by a photoneural system, in which stimulatory signals are generated in the central oscillator located in the suprachiasmatic nucleus (SCN). Stimulation occurs only at night; signals are transmitted to the pineal gland by a neural pathway that passes through central structures, the spinal cord, and the superior cervical ganglia. The pathway terminates in postganglionic superior cervical ganglia fibers that pervade the pineal perivascular space. At night, activation of the SCN → pineal pathway causes the release of norepinephrine (NE), which activates α_{1B}- and β_1-adrenergic

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Abbreviations: AANAT, Arylalkylamine *N*-aceyltransferase; AC, adenylate cyclase; ACTD, actinomycin D; AND gate, a control mechanism requiring synergistic activation of two receptors for full activation; AR, adrenergic receptor; 8-Br, 8-bromo-cAMP; CRE, cAMP-responsive element; CREB, CRE binding protein; DB, dibutyryl-cAMP; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ISO, isoproterenol; LD, light-dark; NE, norepinephrine; PCE, photoreceptor conserved element; pCREB, phosphorylated CREB; PDE, phosphodiesterase; PKA, protein kinase A; PURO, puromycin; qRT, quantitative real-time; Roli, rolipram; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

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receptors (ARs). This dual activation AND gate mechanism stimulates adenylate cyclase (AC) (17), leading to a more than 10-fold spike in cAMP, which is followed by a gradual decline over several hours (18).

SCN stimulation of the pineal gland can be blocked by light acting via a retinohypothalamic projection. This terminates NE release; this, together with rapid uptake of NE, switches off AC activity, leading to a rapid decrease in cAMP levels and reversal of the effects of cAMP (17). These rapid changes are essential for precise effects on biological timing. For example, melatonin production is rapidly terminated when cAMP levels drop because AANAT is immediately inactivated and destroyed, reflecting the reversal of cAMP-dependent binding of AANAT to 14-3-3 protein (19, 20).

A lot is known about the regulation of AC activation in the pineal gland. However, the regulation of cAMP destruction by PDE has received less attention. Whereas it is known that pineal PDE activity exhibits a daily rhythm (21, 22), the molecular basis of this daily rhythm has remained unknown. A clue to the solution of this mystery was provided by results of microarray studies that provided preliminary evidence that the abundance of PDE4B2 mRNA increases at night.[1]

PDE4B2 is one of the 20 known *PDE4* isoforms. There are 11 PDE families, each characterized by differential affinity for cAMP and/or cGMP. The PDE4 family includes four subfamilies (4A, 4B, 4C, and 4D), each encoded by different genes (23, 24). The rat *PDE4B* gene is large and complex (573 kb, 19 exons, National Center for Biotechnology Information Entrez Gene, published as supplemental Fig. 1 on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and encodes four splice variants resulting from selection of alternative promoters. The variants are termed *PDE4B1* (25), *PDE4B2* (25–27), P*DE4B3* (28), and *PDE4B4* (29, 30) (supplemental Fig. 1). The encoded proteins share a common catalytic region but have unique N-terminal sequences. The cellular distribution of some *PDE4B* variant proteins is regulated via posttranscriptional mechanisms including protein-protein interactions mediated by the N-terminal sequences (31, 32). However, PDE4B2, the shortest variant, is devoid of the N-terminal sequences that mediate these interactions; several reports indicate that PDE4B2 mRNA can be modulated experimentally by a cAMP-dependent transcriptional mechanisms involving an intronic promoter (30, 33, 34). The physiological relevance of this has not been established.

Here, we report that PDE4B2 mRNA, PDE4B2 protein, and PDE4 activity increase at night in the rat pineal gland and that PDE4B2 mRNA is notably unstable ($t_{1/2}$, ~20 min). Our results also reveal details of the mechanisms responsible for these changes and their impact on cAMP signaling in the pineal gland. This is of broader interest because it places cAMP regulation of PDE4B2 mRNA and protein in a physiological context.

[1] PDE4B mRNA in the rat pineal gland was 10-fold higher at midnight relative to midday as detected using the Affymetrix RG34A oligonucleotide microarray gene chip, based on analysis of midnight and noon sets of pools of adult rat pineal glands (Humphries, A., D. Carter, R. Baler, S. L. Coon, P. J. Munson, and D. C. Klein, unpublished data).

Materials and Methods

Materials

[8-^{3}H] Adenosine 3′,5′-cyclic phosphate and [^{32}P] α-dCTP were purchased from Amersham Biosciences (Piscataway, NJ). L-(−)-NE, dibutyryl (DB)-cAMP, 8-bromo (8-Br)-cAMP, KT5720, (−)-isoproterenol (ISO), actinomycin D (ACTD), puromycin (PURO), rolipram (Roli), and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO).

Animals and tissue preparations

Rats (male, Sprague Dawley, 150–200 grams) used in organ culture experiments were obtained from Taconic Farms Inc. (Germantown, NY), those used for cell culture were from the University of Alberta animal unit (Edmonton, Canada), and those used for *in situ* hybridization were obtained from the Panum Institute (Copenhagen, Denmark). Animals were housed for 2 wk in light-dark (LD) 14-h light, 10-h dark lighting cycles in all cases except for those animals used for *in situ* hybridization, which were housed in LD, 12 h light, 12 h dark. Animals were killed by CO_2 asphyxiation and decapitated. Tissues were prepared as described previously (5). Animal use and care protocols were in accordance with National Institutes of Health guidelines, with Health Sciences Animal Policy and Welfare Committee of the University of Alberta or the guidelines of European Union Directive 86/609/EEC (approved by the Danish Council for Animal Experiments). To treat animals with ISO, the compound was first dissolved in 0.85% NaCl to a final concentration of 10 mg/ml, and the appropriate volume (20 mg/kg rat) was injected sc at Zeitgeber time (ZT) 4. Rats were killed at ZT7, and their pineal glands were removed and immediately placed on solid CO_2 and stored at −80 C until use.

Organ and cell culture

Rat pineal glands were cultured as described previously (5). Briefly, after 48 h incubation (37 C, 95% O_2, 5% CO_2), the glands were transferred to a top-loading tabletop incubator. The glands were incubated under control conditions for 30 min and then transferred into fresh medium, which in some cases contained a drug of interest. In experiments where mRNA levels, protein levels, and enzyme activities are reported, each analysis was performed on separate sets of pineal glands treated in culture on the same day.

Pinealocytes were prepared as described (35–37). The cells were suspended in DMEM containing 10% fetal bovine serum and maintained at 37 C for 24 h in a gas mixture of 95% air and 5% CO_2 before experimental treatment. Aliquots of cells (2×10^4/0.4 ml) were treated with drugs that had been prepared in concentrated solutions of water or DMSO. The final concentration of the latter never exceeded 1.0%. At this concentration, DMSO had no effect on NE- or ISO-stimulated cAMP or melatonin accumulation. At the end of the treatment period, cells were collected by centrifugation (2 min, 10,000 × *g*, at room temperature), the supernatant was aspirated and saved for the melatonin assay, and the tubes were placed on solid CO_2. The frozen cell pellets were lysed by the addition of 5 mM acetic acid (100 μl) and boiling (5 min). The lysates were stored frozen at −20 C until used for cAMP analysis.

Assays

PDE4B mRNA.

Northern blot (5). Total RNA was separated on a 1.0% agarose/0.7 M formaldehyde gel. Total RNA was transferred to a charged nylon membrane by passive capillary transfer and cross-linked to the membrane using UV light. The hybridization probes used were based either on a common sequence shared by *PDE4B* variants (*PDE4B* nucleotides 1151–1982, GenBank accession no. BC085704; nucleotides 1175–2006, GenBank accession no. L27058) or a *PDE4B2*-specific sequence (nucleotides 131–387, GenBank accession no. L27058). Blots were stripped and reprobed for either glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or 18S rRNA to monitor the quality of the RNA and loading.

For each experiment at least three blots were run, using total RNA extracts from different tissue pools for each lane. The image for each blot was captured using a PhosphorImager (Amersham Biosciences). Signal intensity of the positive band in each lane was normalized to the G3PDH

or 18S signal to correct for variations in loading. All the normalized signals from each blot were then summed to generate a total signal value; each individual value was then expressed as a percentage of total signal to generate a relative intensity value. The data from all blots were compiled and analyzed to generate the data presented in the figures.

Quantitative real-time (qRT)-PCR. Three pools of glands were prepared, each of which was composed of three rat pineal glands. Total RNA (5 μg/gland) was isolated from each pool using the RiboPure RNA isolation kit (Ambion, Austin, TX). The amount and quality of RNA were assessed using a Bio-Rad spectrophotometer (Bio-Rad, Hercules, CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was then subject to deoxyribonuclease treatment using TURBO DNA-*free* (Ambion) to remove contaminating genomic DNA. cDNA production was performed following the Superscript II protocol (Invitrogen, Carlsbad, CA) using 1 μg deoxyribonuclease-treated total RNA as starting material. For the preparation of retinal RNA, a similar procedure was followed in which each sample contained a single retina.

qRT-PCR determinations were made using a LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Reactions (25-μl volume) contained 0.5 μM primers, RT Real-Time SYBR Green master mix (SuperArray Bioscience, Frederick, MD), and cDNA according to the manufacturer's instructions. All incubations included an initial denaturation step at 95 C for 10 min, proceeded by 40 cycles of a 95 C denaturation for 15 sec, 30 sec annealing at 63 C, then extension at 72 C for 30 sec. Primers for each PDE4B variant are as follows: PDE4B1 (GenBank accession no. AF202732), 5′-CAACTGTAAGCCAGGAGTGCT-3′ and 5′-TGGCTTGAGGGTCCAGTG-3′; PDE4B2 (GenBank accession no. L27058), 5′-TAGATGACTGACACCTCATCCCG-3′ and 5′-CTTGCTTCCAAGCTCTTTCTGG-3′; PDE4B3 (GenBank accession no. U95748), 5′-CCCAGTATGCTGCTCTTGCT-3′ and 5′-TCCTTTGGGAAGCCGTGATG-3′; PDE4B4 (GenBank accession no. AF202733), 5′-GGCGACACTCGTGGATATG-3′ and 5′-TGGCTTGAGGGTCCAGTG-3′; PDE4B-common (GenBank accession no. BC085704), 5′-TGAATACATTTCGAACACGTTCTTAG-3′ and 5′-TGCATCAGTTTCTTCACTCCA-3′; and G3PDH (GenBank accession no. BC059110), 5′-TGGTGAAGGTCGGTGTGAACGGAT-3′ and 5′-TCCATGGTGGTGAAGACGCCAGTA-3′. Product specificity was confirmed in initial experiments by agarose gel electrophoresis of the amplified products and thereafter during every qRT-PCR run by melting curve analysis. Typically, approximately 25 cycles were necessary to detect amplification.

Transcript number was determined using internal standards; these were prepared by cloning each variant target PCR product and the G3PDH target PCR product into pGEMT Easy vector (Promega, Madison, WI). Clone verification was performed by direct sequence analysis and also by plasmid DNA digestion followed by agarose gel electrophoresis (2.0%, vol/vol) for visualization of correct product sizes and staining with ethidium bromide (0.5 μg/ml). For each experiment, a set of 100-fold serial dilutions of each internal standard (10^1 to 10^7 copies/1 μl) was prepared and used to generate standard curves. Transcript number was determined using a 2-μl sample of a 10-fold dilution of rat pineal cDNA prepared as described above, and values were normalized to the number of G3PDH copies.

In situ hybridization (5, 38). Sagittal cryostat sections (15 μm) were thawed and fixed for 5 min in 4% paraformaldehyde in PBS, washed two times for 1 min in PBS, and acetylated (0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine; 10 min). The sections were then dehydrated in a graded series of ethanols and delipidated in 100% chloroform (5 min). They were partially rehydrated in 100 and 95% ethanol (1 min each) and allowed to dry.

For hybridization of the cryostat sections, a ^{35}S-labeled 32-mer oligonucleotide probe was prepared based on a rat *PDE4B* sequence (5′-CCAGTGCTGGCGTAGAGAGGAGAACGTGCGTT-3′; antisense strand, nucleotides 1252–1283, GenBank accession no. BC085704), as described previously (5, 38). The labeled probe was diluted in the hybridization buffer (10 μl labeled probe/ml hybridization buffer) consisting of 50% (vol/vol) formamide, 4× standard saline citrate [150 ml mM NaCl, 15 mM sodium citrate (pH 7.0)], 1× Denhardt's solution (0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 10% (wt/vol) dextran sulfate, 10 mM DTT, 0.5 mg/ml salmon sperm DNA, and 0.5 mg/ml yeast tRNA. A 200-μl sample of the labeled probe was placed on each section. The sections were then covered with Parafilm and incubated in a humid chamber overnight at 42 C. After hybridization, the slides were washed in 1× standard saline citrate for 4 × 15 min at 55 C, 2 × 30 min at room temperature, and rinsed twice in distilled water. The sections were dried and either exposed to x-ray film for 1–2 wk or dipped into an Amersham LM-1 emulsion and exposed for 2–4 wk at 4 C. The pineal hybridization signals on x-ray films were quantified using Image 1.42 (Wayne Rasband, National Institutes of Health, Bethesda, MD). Optical density was converted to disintegrations per minute per milligram tissue using simultaneously exposed ^{14}C-standards calibrated by comparison with ^{35}S brain paste standards. Results are based on the analysis of pineal glands from four animals killed at night and four during the day.

PDE4B protein. Western blots were performed as previously described (5) using an anti-PDE4B selective serum (PD4-212AP; FabGennix Inc., Frisco, TX). Briefly, samples containing 60 μg protein was resolved on preformed 10% Tris/glycine (1 mm) gels using the manufacturer's protocol (Novex, San Diego, CA). The proteins were electroblotted onto an Immobilon-P (0.45 μm) transfer membrane in a semidry blotting system. Anti-PDE4B serum was diluted (1:1000) in PBS containing 1 mg/ml BSA fraction V and 0.05% thimerosal.

Western blot images were analyzed as described above for Northern blot images except that signals were not corrected for variations in loading.

PDE4 activity. PDE4 activity was assayed using a PDE4 Enzymatic Assay Kit (FabGennix Inc.); the final concentration of cAMP was 2 μM. Pineal homogenates (10 μg protein/assay) were assayed for total and Roli (10 μM) resistant PDE activity; PDE4 activity was estimated from the difference between total and Roli-resistant PDE activity (39).

cAMP (36, 40, 41). Samples of the pineal homogenate supernatant were used to determine cellular cAMP content, using an RIA procedure in which samples were acetylated before analysis. Because there was a small batch-to-batch variation of the cyclic nucleotide responses between cell preparations, all statistical comparisons were performed within the same batch of cells.

Melatonin (42). Melatonin was extracted from 350 μl pinealocyte cell suspension by vortexing with 1 ml methylene chloride followed by centrifugation. After centrifugation, the organic phase was collected and evaporated to dryness. The residue was reconstituted in 500 μl assay buffer [0.01 M phosphate buffer (pH 6.5) containing 0.1% gelatin]. The recovery of melatonin was more than 98%. The extracted melatonin was assayed by RIA.

Statistical analysis

Data are expressed as mean ± SE values for the number of determinations indicated. Statistical analyses were performed using Student's *t* test or Mann-Whitney *U* test for two groups and ANOVA for multiple groups.

Results

A daily rhythm in pineal PDE4B mRNA reflects a rhythm in the PDE4B2 variant

PDE4B mRNA is approximately 5-fold more abundant in the pineal gland at night than during the day, as indicated by both Northern blot and qRT-PCR (Fig. 1), confirming preliminary microarray results (see Footnote 1). Most of the PDE4B transcripts are the 4B2 variant; the levels of 4B1, 4B3, and 4B4 transcripts are relatively insignificant compared with 4B2 transcripts (Fig. 1B). The maximal nocturnal increase in PDE4B2 mRNA is approximately 5-fold and occurs with a doubling time of less than 2 h; the halving time during the night from the peak is approximately 3 h according to Northern blot analysis and approximately 5 h according to qRT-PCR. This difference is likely due to the greater linear range of the qRT-PCR method. These results establish that

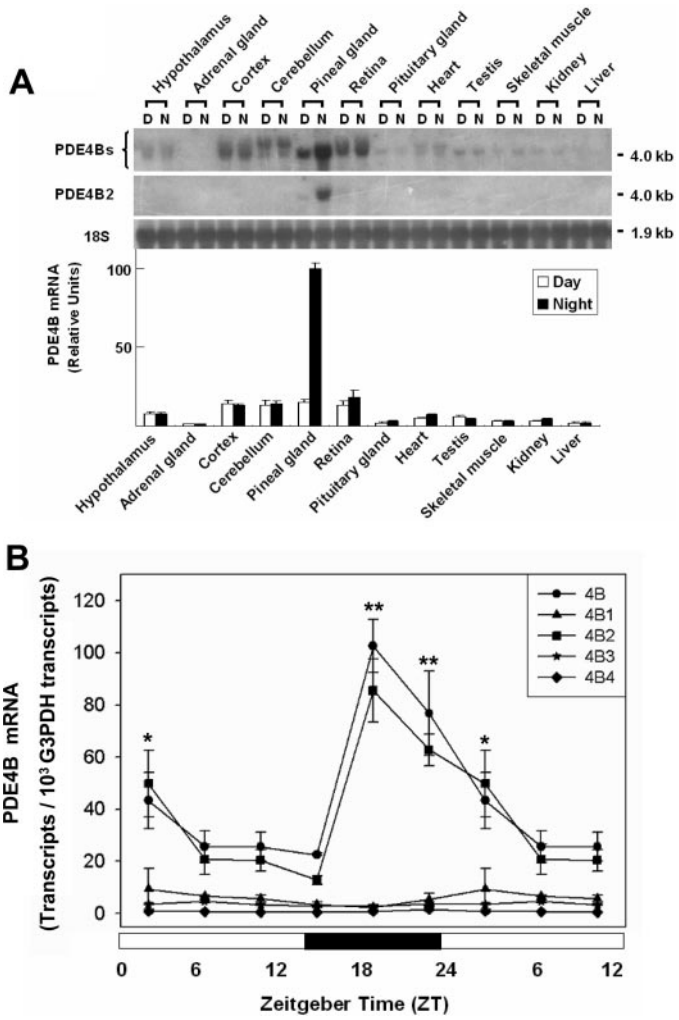

FIG. 1. Analysis of PDE4B mRNA in the pineal gland and other tissues. A, PDE4B and PDE4B2 mRNA in selected tissues. Rats were housed in a controlled lighting environment (LD, 14 h light, 10 h dark). Total RNA was obtained from day tissues removed at ZT7 and night tissues removed at ZT19 under dim red light. RNA preparation and Northern blot analysis were performed as described in *Materials and Methods*. The blot was hybridized with a rat PDE4B probe directed against sequences common to all isoforms. The blot was stripped and hybridized with a rat PDE4B2-specific probe. To normalize for RNA loading, blots were stripped and reprobed with 18S rRNA. D, Day; N, night. Results are presented as the mean ± SE of three replicates. B, PDE4B splice variants in the pineal gland. Pineal glands were obtained at the indicated times, and qRT-PCR was done as detailed in *Materials and Methods*. The ZT3, 7, and 11 data points are double plotted. Results are presented as the mean ± SE of three replicates. *, Statistically different from the ZT15 group, $P < 0.05$; **, statistically different from the ZT15 group, $P < 0.01$ *vs.* ZT15 group. For further details, see *Materials and Methods*.

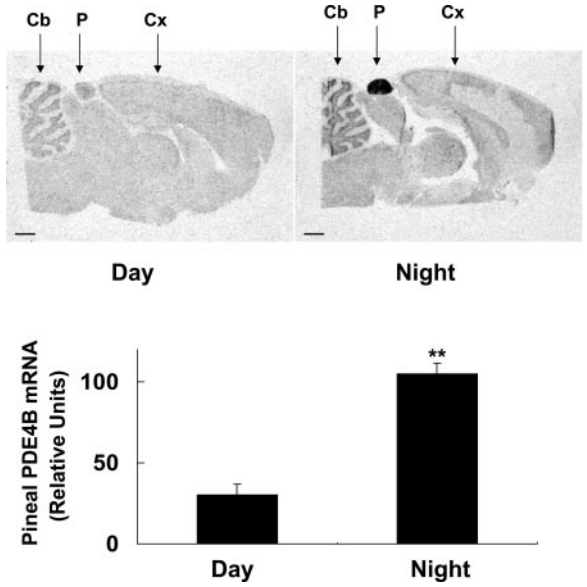

FIG. 2. *In situ* hybridization analysis of the distribution of PDE4B mRNA in the rat brain. Animals were housed in a controlled lighting environment for 2 wk (LD, 12 h light, 12 h dark). *Top left*, Sagittal section through the pineal gland from an animal killed during daytime (ZT6); *top right*, similar section from an animal killed during nighttime (ZT18). *Bars*, 1 mm. Each *bar* in the *bottom* panel presents the quantitation of the strength of the hybridization signal in four pineal glands; for each pineal gland, four sections were analyzed. Results are presented as the mean ± SE. Cb, Cerebellum; P, pineal gland; Cx, cortex. **, Significantly greater than day values, $P < 0.01$. For further details, see *Materials and Methods*.

pineal PDE4B2 mRNA exhibits a 24-h rhythm in abundance, with high values at night.

Examination of PDE4B mRNA in other tissues by Northern blot (Fig. 1A), *in situ* hybridization (Fig. 2), and qRT-PCR (supplemental Fig. 2) reveals that nocturnal pineal PDE4B2 mRNA levels are more than 6-fold greater than in other tissues except lung, in which expression is approximately 50% that in the night pineal gland. PDE4B2 mRNA is found in nonpineal tissues including the retina; however, no tissue has levels as high as those in the night pineal gland (Figs. 1–3, supplemental Figs. 2–4). *In situ* hybridization analysis confirmed this and also revealed that the PDE4B signal is consistently strong in all parts of the gland, indicative of localization in the pinealocyte. A moderately strong hybridization signal is also seen in the Purkinje cell layer and granular layer of the cerebellum as well as in the neocortex with a maximum intensity in the granular layer II (Fig. 2). These studies highlight the high level of expression of *PDE4B2* in the pineal gland relative to other brain regions.

The expression of *PDE4B* variants in the retina was examined because many elements linked to melatonin synthesis and phototransduction are expressed in both tissues (43). The total copy number of all PDE4B mRNA variants in the retina is approximately 10% of that in the pineal gland, indicating that expression of *PDE4B* is stronger in the pineal gland. In addition, the most highly expressed *PDE4B* variant in the retina is 4B3, not 4B2, as it is in the pineal gland; in the retina, the abundance of the 4B2 variant in the retina is approximately 2.5% of that in the pineal gland (Fig. 1; supplemental Fig. 3). In addition, rhythmic changes in *PDE4B*-derived transcripts are 180° out of phase the pineal PDE4B2 rhythm. Accordingly, the expression profiles of *PDE4B*-derived mRNAs in the pineal gland and retina are distinctly different.

PDE4B2 protein and enzyme activity follow changes in pineal PDE4B2 mRNA

Western blot analysis revealed that a single 65-kDa band of PDE4B-immunopositive protein was present in the pineal

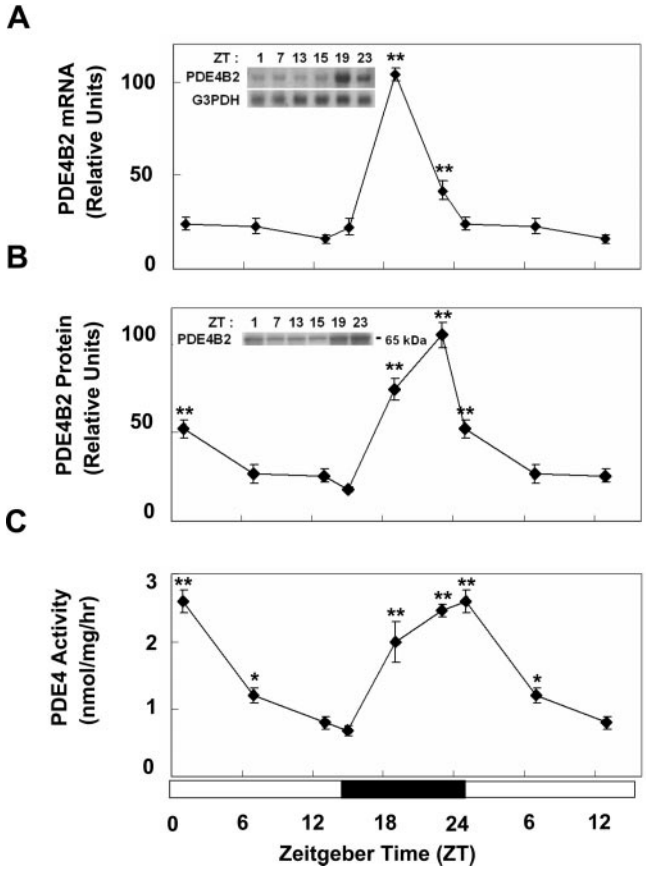

FIG. 3. The daily rhythms in rat pineal PDE4B2 mRNA, PDE4B2 protein, and in PDE4 activity. Animals were housed in a controlled lighting environment for 2 wk (LD, 14 h light, 10 h dark), pineal glands were obtained at the indicated times, and PDE4B2 mRNA, PDE4B2 protein, and PDE4 activity were subsequently measured to characterize the daily changes in these parameters. The ZT1, 7, and 13 data points are double plotted. Results are presented as the mean ± SE of three replicates. A, PDE4B2 mRNA: Northern blot analysis was done using 8 μg total RNA/lane. To normalize for RNA loading, the blot was reprobed for G3PDH mRNA. B, PDE4B2 protein. Western blot analysis was done using 60 μg soluble pineal protein/lane. After electrophoresis, proteins were transferred and immunodetected using an anti-PDE4B. The 65-kDa size of the immunopositive band of protein corresponds to that of PDE4B2 protein and not to larger PDE4B splice variants (44). C, PDE4 activity. PDE4 activity represents Roli-sensitive PDE activity, *i.e.* the difference between total PDE activity and PDE activity in the presence of Roli (supplemental Fig. 5). *, Statistically different from the ZT15 group, $P < 0.05$. **, Statistically different from the ZT15 group, $P < 0.01$. For further details, see *Materials and Methods*.

gland at night, corresponding to the PDE4B2 protein (44). The abundance of PDE4B2 protein changes in a pattern similar to that of pineal PDE4B2 mRNA (Fig. 3B), gradually reaching an approximately 6-fold increase in abundance near the end of the night period. In addition, a daily rhythm in PDE4 activity also occurs in the pineal gland (Fig. 3C). The more prolonged elevation of PDE4B2 protein and activity levels relative to mRNA suggests the protein has greater stability than the transcript. The analysis of PDE4 activity (Fig. 3C) was performed using the PDE4-selective inhibitor Roli (39, 45, 46). PDE4 was found to represent 20% of total PDE activity at ZT15 and 46% of total PDE activity at ZT1 (supplemental Fig. 5), reflecting the presence of other forms of PDE. These results are consistent with the conclusion that the nocturnal increase in total PDE activity reflects the increase in PDE4 activity due to the increase in PDE4B2 protein.

Together, these observations indicate that PDE4B2 mRNA, PDE4B2 protein, and PDE4 activity are elevated during the latter half of the dark period and that the changes seen in PDE4B2 protein and PDE4 activity lag behind those of the mRNA.

PDE4B2 mRNA is under photoneural and circadian control

The increase in PDE4B2 mRNA at night suggests this may be dependent upon exposure to darkness, as is the case with the expression of many pineal genes controlled by the SCN → pineal neural system (2, 5, 10, 11, 14, 16). SCN → pineal stimulation can be blocked by light. Here, we blocked SCN stimulation of the pineal gland by prolonging the light period (Fig. 4A, night, PL) and found this blocked the increase in PDE4B2 mRNA. This is consistent with the conclusion that PDE4B2 mRNA levels are regulated by the SCN → pineal neural pathway.

The decrease in PDE4B2 mRNA ($t_{1/2} \sim 4$ h) during the last half of the night suggests that PDE4B2 mRNA is relatively unstable. Here, we reexamined this by terminating SCN stimulation of the pineal gland with a 30′ light pulse at night (Fig. 4A, night, LP). This resulted in a very rapid ($t_{1/2} \sim 20$ min) decrease ($P < 0.01$ compared with the night group) in PDE4B2 mRNA.

We also tested the hypothesis that the 24-h rhythm in PDE4B2 mRNA was driven by an endogenous clock by housing animals in constant darkness for 4 d (Fig. 4B, DD). Under these conditions, the rhythm in PDE4B2 mRNA persisted, indicating that it does not require dark/light transitions and is driven by an endogenous circadian clock. We also found that the rhythm was absent in animals maintained in constant light (Fig. 4B, LL), which supports the conclusion that light blocks SCN stimulation of the pineal gland.

PDE4B2 mRNA, PDE4B2 protein, and PDE4 activity are controlled by an adrenergic-cAMP mechanism

The adrenergic regulation of PDE4B2 mRNA was examined using adrenergic agonists. *In vivo* studies revealed that treatment with ISO increases PDE4B2 mRNA and protein (Fig. 5). *In vitro* studies were performed using rat pineal glands that had been cultured for 48 h to allow nerve endings to degenerate; this eliminates the reuptake effects of nerve endings that reduce NE stimulation of pinealocytes (47). Glands were treated with a concentration of NE that produces submaximal/maximal induction of other genes in the pineal gland (48); this increased PDE4B2 mRNA, PDE4B2 protein, and PDE4 activity (Fig. 6), supporting the interpretation that PDE4B2 mRNA, protein, and activity are controlled by NE and that this mechanism explains the increase in PDE4B2 mRNA that occurs at night.

We then investigated whether an established second messenger of NE in the pineal gland, cAMP, mediates control of *PDE4B2* expression. This was done by treating glands with one of two cAMP protagonists, DB-cAMP or 8-Br-cAMP (5, 49). These agents can act as cAMP mimics or as inhibitors of

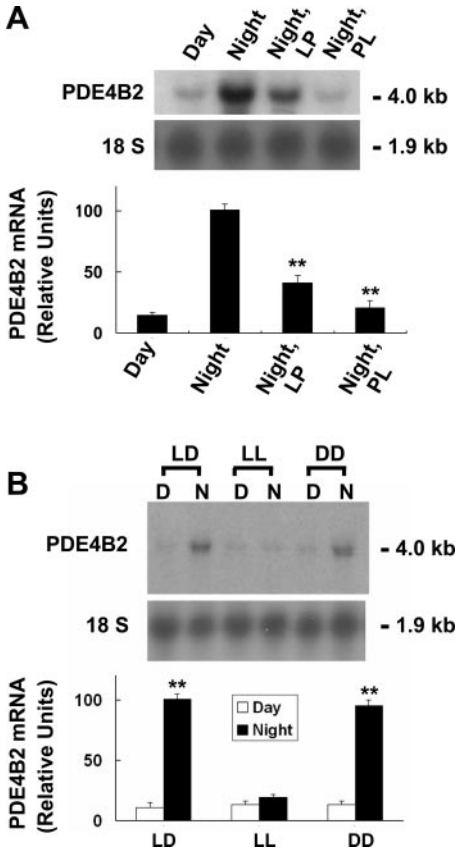

FIG. 4. Rat pineal PDE4B2 mRNA levels are regulated by environmental lighting and a circadian clock. Rats were housed in a controlled lighting environment (LD, 14 h light, 10 h dark) for 2 wk. A, On the day of the experiment, day (ZT7) and night (ZT19) pineal glands were obtained. In addition, one group of animals was exposed to a 30′ light pulse (LP) initiated at ZT1830; their pineal glands were removed at ZT1900. Exposure to the 30′ light pulse reduced PDE4B2 mRNA levels to more than 50% night values. Another group of animals was exposed to light during the night. This prolongation of lighting (PL) prevented the nocturnal increase in PDE4B2 mRNA. Results are mean ± SE of three replicates. **, Statistically lower than the night group, $P < 0.01$. B, Pineal glands were collected at ZT7 and ZT19 from rats under normal lighting (LD), constant light (LL), and constant darkness (DD) conditions. Results are mean ± SE of three replicates. **, Statistically greater than day group, $P < 0.01$. For further details, see *Materials and Methods*.

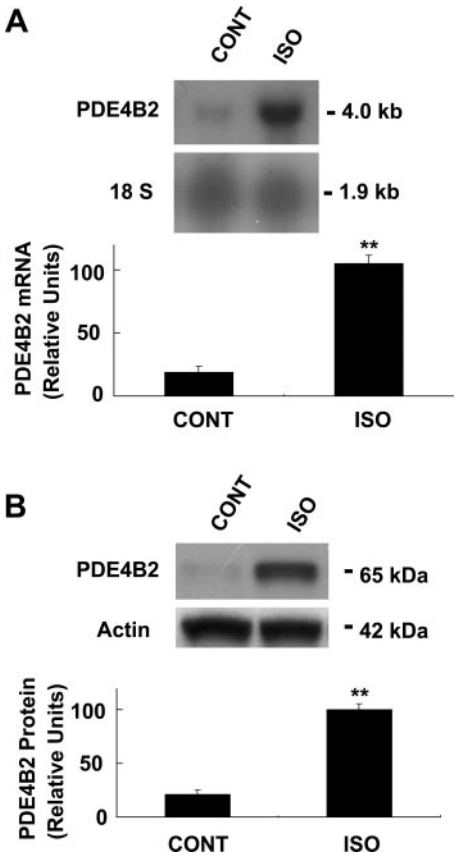

FIG. 5. *In vivo* β-adrenergic treatment elevates pineal PDE4B2 mRNA and protein. Rats were injected sc in the nape of the neck with a solution of ISO (20 mg/kg in 0.4 ml of 0.85% NaCl) at ZT4. Control and treated animals were killed at ZT7; their pineal glands were then removed and stored on solid CO_2. A, PDE4B2 mRNA. Northern blots were prepared and analyzed using a PDE4B2-selective probe. Each lane was loaded with 8 μg total RNA obtained from a pool of two pineal glands. To normalize for variations in RNA loading, the blot was reprobed for 18S rRNA. The results were confirmed in three independent experiments. Results are mean ± SE of three replicates. B, PDE4B protein. For Western blot analysis, each lane was loaded with 60 μg protein from a pineal extract. Immunodetection was done using an anti-PDE4B serum. Results are presented as the mean ± SE of three replicates. **, Statistically different from the CONT group, $P < 0.01$. For further details, see *Materials and Methods*.

PDE activity or as both. The doses selected produce maximal or near-maximal induction of other genes in the pineal gland (2, 10, 13, 15, 16, 50, 51). Treatments with these agents elevated PDE4B2 mRNA, PDE4B2 protein, and PDE4 enzyme activity. In contrast, treatment with the protein kinase C agonist phorbol 12-myristate 13-acetate did not affect these parameters (Fig. 6). These findings together with the known effects of NE and ISO on pineal cAMP levels indicate that NE acts through a cAMP mechanism to control the abundance of PDE4B2 mRNA.

cAMP regulates the expression of *AANAT* and other genes in the pineal gland through activation of protein kinase A (PKA), which is thought to act through subsequent phosphorylation of CRE binding protein (CREB) (2, 5, 17, 52). To investigate the possible involvement of PKA in the effects of cAMP on the abundance of PDE4B2 mRNA, glands were treated with the PKA inhibitor KT5720, using a dose known to block NE-induction of other pineal genes (5, 53). Treatment with this agent was initiated 1 h before NE treatment; this blocked the NE-stimulated increase of PDE4B2 mRNA and protein (Fig. 7; $P < 0.01$ compared with the NE-treated group). These findings support the conclusion that pineal PDE4B2 mRNA is increased by a NE/cAMP/PKA mechanism.

Induction of PDE4B2 mRNA requires de novo transcription but not de novo protein synthesis

The issue of whether NE-dependent elevation of PDE4B2 mRNA requires ongoing transcription was addressed by treating cultured rat pineal glands with NE in the presence or absence of an inhibitor of mRNA synthesis, ACTD (Fig. 8). This inhibited the expected increase in PDE4B2 mRNA, in-

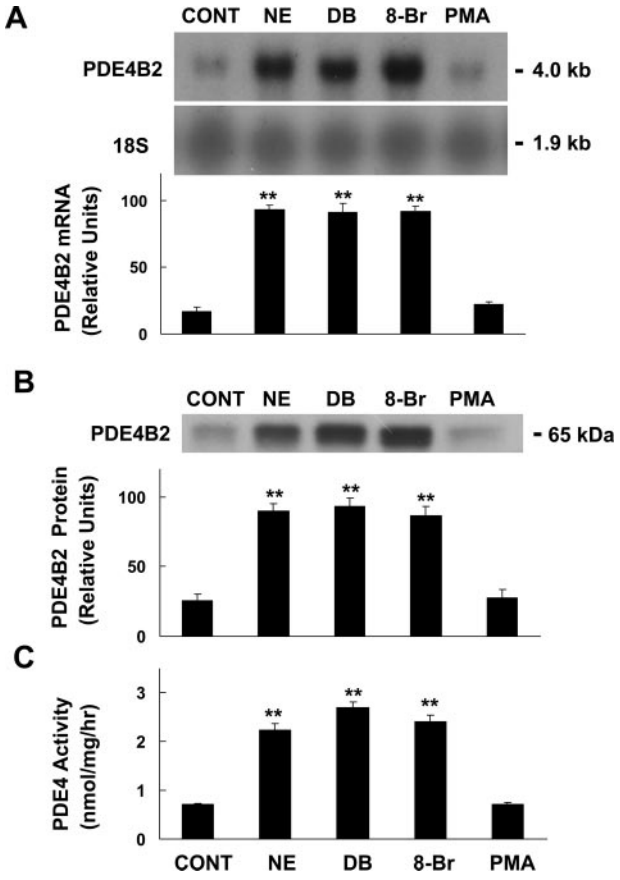

FIG. 6. Pineal PDE4B2 mRNA, protein levels, and PDE4 activity are elevated in organ culture by treatment with NE or cAMP protagonists. Pineal glands were incubated for 48 h and then treated with 1 μM NE, 500 μM DB, 500 μM 8-Br, 1 μM phorbol 12-myristate 13-acetate, or no drug (CONT) for 6 h. At the end of treatment, glands were placed on solid CO_2 and stored at −80 C. Results are presented as the mean ± SE of three replicates. A, PDE4B2 mRNA. For Northern blot analysis, each lane contained 6 μg total RNA. Treatments with NE, DB, and 8-Br significantly elevated PDE4B2 mRNA. B, PDE4B2 protein. For Western blot analysis, each lane was loaded with 60 μg protein from a pineal extract. Treatments with NE, DB, and 8-Br significantly elevated PDE4B2 protein. C, PDE4 enzyme activity. Treatments with NE, DB, and 8-Br significantly elevated PDE4 activity. **, Statistically greater than CONT group, $P < 0.01$ For further details, see *Materials and Methods*.

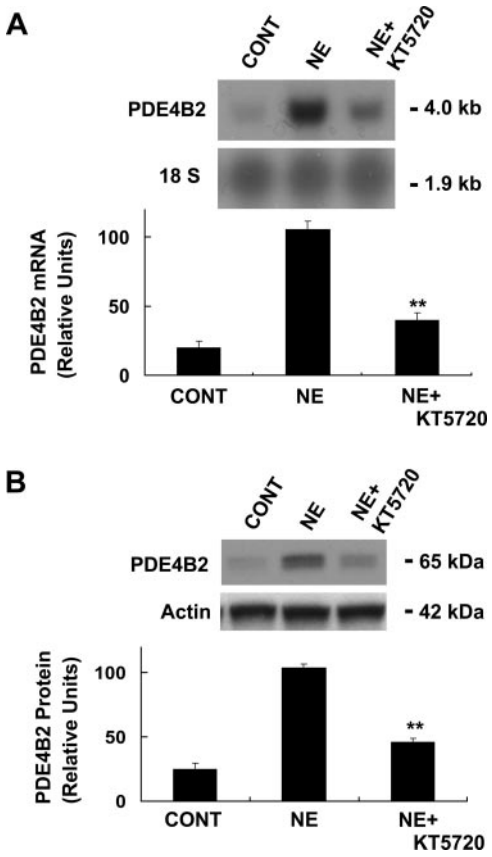

FIG. 7. The PKA antagonist KT5720 blocks NE elevation of pineal PDE4B2 mRNA and protein in organ culture. Pineal glands were treated with 3 μM KT5720 for 1 h after a 48-h incubation period; NE (1 μM) was then added to the medium, and the incubation was continued for 6 h. A, PDE4B2 mRNA. For Northern blot analysis, each lane contains 6 μg total RNA obtained from a pool of four pineal glands. After hybridization with the PDE4B2-selective probe, the blot was stripped and reprobed with 18S rRNA probes as a control. Results were confirmed in three independent experiments. B, PDE4B2 protein. For Western blot analysis, each lane was loaded with 60 μg protein from a pineal extract. Results are mean ± SE of three replicates. **, Statistically lower than the NE-treated group, $P < 0.01$. For further details, see *Materials and Methods*.

dicating that the elevation in PDE4B2 mRNA requires transcription.

In addition, we examined whether protein synthesis was essential for the increase in PDE4B2 mRNA to occur. This was of special interest because the time course for the increase in PDE4B2 mRNA was somewhat slower than that reported for other NE/cAMP-regulated transcripts, including AANAT (2), Fra-1 (50), MKP-1 (16), and MAT2a (5), raising the possibility that this delay reflected the time required for *de novo* synthesis of an protein essential for this response (*e.g.* a transcription factor). However, PURO-induced inhibition of protein synthesis (2, 5) (Fig. 8) did not block the NE-induced PDE4B2 mRNA elevation. This indicates that the effects of NE/cAMP/PKA elevation of PDE4B2 mRNA do not require *de novo* protein synthesis.

Negative feedback: inhibition of PDE4 modulates pineal cAMP and melatonin during the late phase of activation

Based on the above studies, it appears that PDE4 activity increases gradually over several hours after the initiation of adrenergic stimulation. Based on this, one would predict that changes in PDE4 activity would not impact cAMP levels during the early phase of the NE response, but only during the late phase. This prediction was tested using the PDE4-selective inhibitor Roli. It did not alter the spike in cAMP induction seen immediately after treatment of rat pinealocytes with NE; however, Roli treatment did have a significant effect 2 h after treatment, which increased at 4 h and was sustained for 8 h (Fig. 9), which coincides with the elevation of PDE4B protein and PDE4 activity.

To examine the effects of PDE4 inhibition on melatonin production, a paradigm was used that tested whether Roli administration could alter the decline of melatonin, which occurs when adrenergic stimulation of cultured pinealocytes

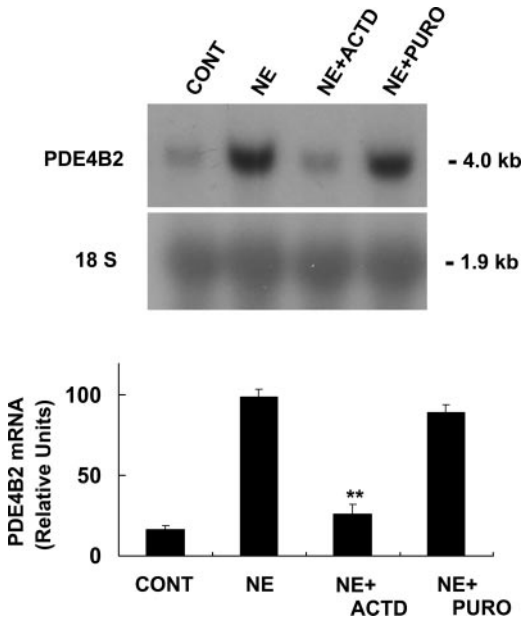

FIG. 8. NE-induced elevation of pineal PDE4B2 mRNA requires gene transcription but not protein synthesis. Pineal glands were treated with 30 μg/ml ACTD or 50 μg/ml PURO for 1 h and during the subsequent 6-h treatment with 1 μM NE. Each lane was loaded 6 μg total RNA obtained from a pool of four pineal glands. After hybridization with the PDE4B2 probe, the blot was stripped and reprobed with 18S rRNA probe as a control. Results were confirmed in three independent experiments. The values are mean ± SE of three replicates. **, Statistically lower than NE-treated group, $P < 0.01$. For further details, see *Materials and Methods*.

is blocked. To do this, cells were treated with NE for 6 h, which elevated melatonin production and PDE4B2 protein. Cells were then collected and resuspended in media containing the β-AR antagonist propranolol to terminate adrenergic activation; Roli was also present in some cultures (Fig. 10). Roli treatment had significant effects on melatonin production, resulting in 3-fold greater levels. These results indicate that the increase in PDE4B2 protein impacts cAMP-regulated effects.

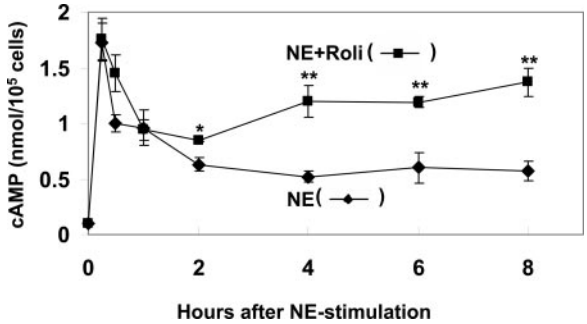

FIG. 9. Effects of the PDE4 inhibitor Roli on NE-induced cAMP elevation in pinealocytes. Pinealocytes were incubated in DMEM and stimulated with 0.3 μM NE or 0.3 μM NE and 10 μM Roli. The lysates were centrifuged (12,000 × *g*, 10 min, 4 C), and samples of the supernatant were used to determine intracellular cAMP levels. Values are presented as the mean ± SE of three replicates. *, Statistically lower than the NE group, $P < 0.05$; **, statistically lower than the NE-treated group, $P < 0.01$. For further details, see *Materials and Methods*.

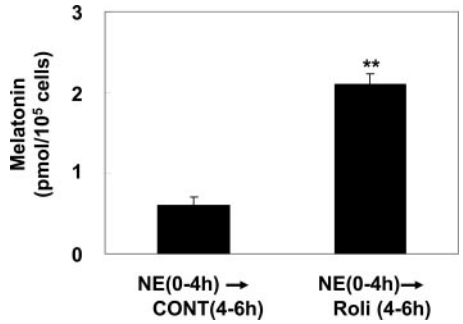

FIG. 10. Effects of the PDE4 inhibitor Roli on melatonin in cultured pinealocytes. Pinealocytes were stimulated with 1 μM NE for 4 h to elevate melatonin production. NE stimulation was terminated by replacing the media with media containing the β-adrenergic antagonist propranolol (3 μM). As indicated, some of the cultures were treated (2 h) with Roli (10 μM) dissolved in DMSO or only with DMSO (CONT). Melatonin was assayed by RIA. Values are presented as the mean ± SE of three replicates. **, Statistically greater than the control group, $P < 0.01$. For further details, see *Materials and Methods*.

Discussion

A rhythm in pineal PDE activity was first reported by Minneman and Iversen (21) and subsequently confirmed by Epplen *et al.* (22). The findings here extend this line of investigation by revealing that the increase in total pineal PDE activity primarily reflects an increase in the contribution from PDE4 activity, reflecting the selective increase in PDE4B2 protein. Moreover, our results establish that the nocturnal increase in PDE4 activity reflects the adrenergic/cAMP/PKA elevation of PDE4B2 mRNA. Similar dynamics have been described in which PDE4B2 mRNA was examined in cultured cortical neurons, establishing that cAMP controls PDE4B2 mRNA through effects mediated by a phosphorylated CREB (pCREB)/cAMP-responsive element (CRE) mechanism (44). The results of the present study are in agreement with the conclusion that the same cAMP-dependent mechanism controls PDE4B2 mRNA in the pineal gland and that the CREs mediating these effects are located in an alternative intronic promoter in the rat *PDE4B* gene (Fig. 11; supplemental Fig. 6) (27, 44). It should be noted that cAMP induction of *AANAT* in the pineal gland involves a mechanism dependent upon promoter domains containing both CRE elements and CRE/CCAAT complexes; other induced genes also contain these complexes (10, 12, 50). Such complexes also occur within the intronic promoter region of the rat *PDE4B* gene (supplemental Fig. 6) as revealed by *in silico* analysis (Genomatix Software, http://www.genomatix.de).

Our finding that PDE4B2 mRNA levels are many fold higher in the rat pineal gland than in most other tissues examined may be explained in part by CRE elements and the CRE/CCAAT complex and in part by the four photoreceptor conserved element (PCE)-related sequences in the intronic promoter, located within approximately 1 kb of the translation start site (supplemental Fig. 6). PCE elements appear to participate in rhythmic and tonic expression of a subset of genes expressed nearly exclusively in the pineal gland and retina, which are involved in melatonin synthesis and phototransduction (54, 55). Two transcription factors that activate transcription through binding to PCE sites are orthodenticle homeobox protein Otx2 and cone-rod homeobox

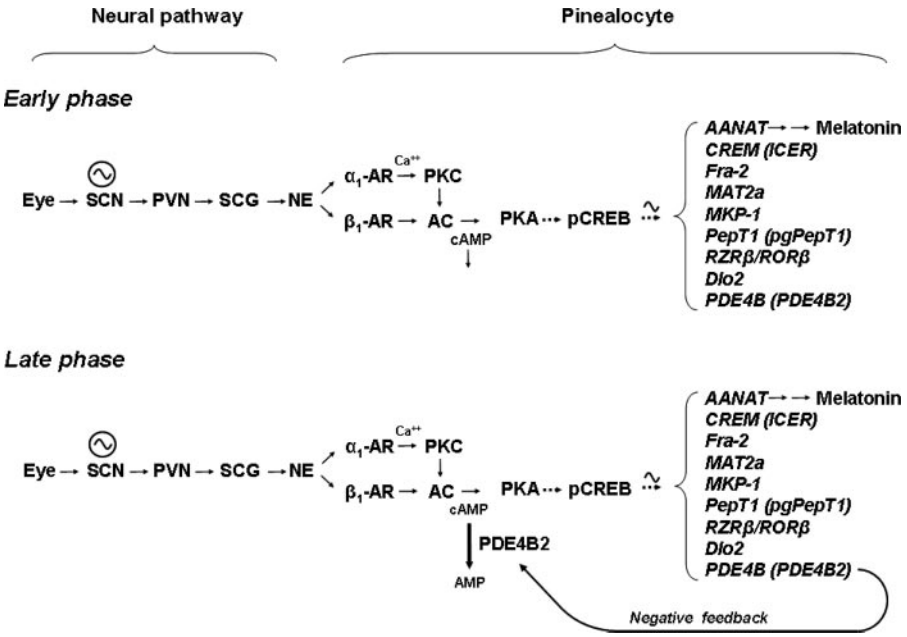

FIG. 11. Early and late phases of the adrenergic/cAMP stimulation of the rat pineal gland. During the early phase of adrenergic stimulation of the pineal gland, signals originating in the SCN and transmitted to the pineal gland by a neural circuit release NE from sympathetic nerves in the pineal gland. NE acts through an adrenergic AND gate mechanism to elevate cAMP (18, 63, 64). As a result, transcription of the indicated genes increases, including *AANAT*, which is required for melatonin production to increase. The role of pCREB has been established in the case of expression of *AANAT* (52) in the rat pineal gland and of *PDE4B* in rat cortical cells (44). The role of pCREB in the control of the other genes has not been established, as indicated by the *dashed arrow*. cAMP-induced expression of *CREM*, *PepT1*, and *PDE4B* in the pineal gland results in generation of the variants identified in *parentheses*; this is mediated by alternative promoters. During the late phase, PDE4B2 protein accumulates, thereby elevating PDE4 activity and enhancing cAMP degradation. The cAMP-induced down-regulation of cAMP signaling via elevation of PDE4B2 protein represents negative feedback in this system.

protein Crx (55, 56), both of which are highly expressed in the pineal gland (57) Accordingly, we suspect that the high levels of PDE4B2 mRNA in the pineal gland reflect activation mediated by CRE sites, the CRE/CCAAT complex and the four PCE-related sites; further experimental testing may confirm this suspicion.

It is of interest that high levels of PDE4B2 do not occur in the retina as is the case for many signal transduction genes expressed in the pineal gland (Fig. 1; supplemental Figs. 2 and 3). This may be due in part to the absence or low level of activity of the cAMP/PKA/CREB mechanism in the rat retina (58), where daily changes in *AANAT* expression are due to a circadian E box mechanism and not a cAMP/PKA/CREB mechanism. Although low levels of PDE4B2 and PDE4B3 transcripts were detected in the rat retina, they did not exhibit an increase during the night but increased during daytime, 180° out of phase with the pineal PDE4B2 mRNA rhythm. This inverse relationship is likely to reflect differences in cellular context and in the mechanisms that control synthesis of each PDE4B variants.

The observation that changes in PDE4B2 mRNA are translated with little delay into changes in PDE4B2 protein indicates that transcription is a major factor controlling the abundance of PDE4B2 protein and PDE4 activity in the pineal gland. This is not the case with other PDE4B variants because the major factor controlling them appears to be protein-protein interactions that target them to specific sites in the cell (31, 32). Although the same targeting may not be possible with the PDE4B2 variant because it lacks the interacting N-terminal sequences, it is possible that other sequences might mediate intracellular localization/targeting (34) of PDE4B2 protein. Accordingly, the impact that PDE4B2 has on cAMP degradation would reflect both the abundance of the protein and where it is concentrated as a result of targeting. Such targeting could profoundly and selectively alter local concentrations of cAMP in critical regulatory microdomains without having a major impact on cAMP levels in other compartments (31, 32). Perhaps future research will reveal a targeting function conferred by the PDE4B2 N-terminal region.

The time course of the PDE4B2-dependent increase in PDE4 activity is of interest because it relates to the regulation of intracellular cAMP levels and downstream events. The elevation of cAMP in the pineal gland reflects very rapid AR-dependent activation of AC (18). In contrast, the effect of adrenergic elevation of PDE4 activity has a time course of hours, reflecting the time required for transcription and translation. Accordingly, it appears that adrenergic activation of AC plays a dominant role in initiating the rapid increase in cAMP in the early phase of the adrenergic response and that during the late phase, regulation is influenced both by AC and PDE (Fig. 11); apparently, the contribution from PDE4B2 influences the intensity of the cAMP response and the rate at which cAMP is destroyed after cessation of adrenergic stimulation of AC.

A link between *PDE4* and circadian biology has been suggested previously by the studies of Masumoto *et al.* (59), who reported that inhibition of PDE4 increases SCN expression of *Per1*, a molecular component of the mammalian circadian clock. Prosser and Gillette (60) have also shown *in vitro* that cAMP can reset the mammalian circadian clock in the SCN. These findings together with those presented in this report provide reason to entertain the idea that PDE4B2 may play a special role in circadian biology, perhaps enabled by the unstable natures of the PDE4B2 mRNA and protein that allow large changes in PDE4 activity to occur at a rate consistent with 24-h changes in function.

The unstable nature of PDE4B2 mRNA deserves special mention; here, we found that after levels were elevated at night, exposure to light caused a rapid decrease ($t_{1/2} \sim 20$ min). This is unusual because most transcripts in the pineal gland do not exhibit a significant night/day difference (see

Footnote 1). However, those that do may also exhibit such rapid changes. The specific nature of the decrease in PDE4B2 is consistent with a mechanism involving micro-RNA-directed destruction, a possibility that deserves further investigation.

Although the mechanisms of down-regulation in the pineal gland have not been detailed, it is well known that multiple mechanisms mediate down-regulation of receptor-dependent activation of second messenger synthesis in many systems. The present study highlights cAMP degradation as an important component of cAMP metabolism in the pineal gland, a topic that has received little recent attention (61). Our results suggest that cAMP-dependent elevation of PDE4B2 mRNA is a significant factor that is likely to broadly influence pineal function, based on the important effects of cAMP in this tissue (Fig. 11). We suspect that many mechanisms influence pineal signal transduction (62) and that adrenergic/cAMP control of PDE4B2 protein is only one of many elements in a complex negative feedback system that controls the duration and intensity of the response of the pineal gland to neural stimulation, thereby contributing to the precision and reliability of pineal responses and biological timing.

Note Added in Proof

Adrenergic/cAMP activation of the pineal gland has been found to result in phosphorylation of histone H3 (65). This raises the possibility that cAMP/PKA-dependent phosphorylation of histone H3 is broadly involved in control of gene expression in the pineal gland, including the cAMP/PKA-dependent induced expression of *PDE4B2* reported here.

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References

1. **Arendt J** 1998 Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. Rev Reprod 3:13–22
2. **Roseboom PH, Coon SL, Baler R, McCune SK, Weller JL, Klein DC** 1996 Melatonin synthesis: analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. Endocrinology 137:3033–3045
3. **Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R** 1997 The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 52:307–357
4. **Klein DC** 12 December 2006 Arylalkylamine N-acetyltransferase. "The timezyme." J Biol Chem 10.1074/jbc.R600036200.
5. **Kim JS, Coon SL, Blackshaw S, Cepko CL, Moller M, Mukda S, Zhao WQ, Charlton CG, Klein DC** 2005 Methionine adenosyltransferase:adrenergic-cAMP mechanism regulates a daily rhythm in pineal expression. J Biol Chem 280:677–684
6. **Banik U, Wang GA, Wagner PD, Kaufman S** 1997 Interaction of phosphorylated tryptophan hydroxylase with 14-3-3 proteins. J Biol Chem 272:26219–26225
7. **Klein DC, Ganguly S, Coon SL, Shi Q, Gaildrat P, Morin F, Weller JL, Obsil T, Hickman A, Dyda F** 2003 14-3-3 Proteins in pineal photoneuroendocrine transduction: how many roles? J Neuroendocrinol 15:370–377
8. **Guerrero JM, Reiter RJ** 1992 Iodothyronine 5′-deiodinating activity in the pineal gland. Int J Biochem 24:1513–1523
9. **Tanaka K, Murakami M, Greer MA** 1986 Type-II thyroxine 5′-deiodinase is present in the rat pineal gland. Biochem Biophys Res Commun 137:863–868
10. **Gaildrat P, Moller M, Mukda S, Humphries A, Carter DA, Ganapathy V, Klein DC** 2005 A novel pineal-specific product of the oligopeptide transporter PepT1 gene: circadian expression mediated by cAMP activation of an intronic promoter. J Biol Chem 280:16851–16860
11. **Baler R, Klein DC** 1995 Circadian expression of transcription factor Fra-2 in the rat pineal gland. J Biol Chem 270:27319–27325
12. **Pfeffer M, Maronde E, Molina CA, Korf HW, Stehle JH** 1999 Inducible cyclic AMP early repressor protein in rat pinealocytes: a highly sensitive natural reporter for regulated gene transcription. Mol Pharmacol 56:279–289
13. **Baler R, Coon S, Klein DC** 1996 Orphan nuclear receptor RZRβ: cyclic AMP regulates expression in the pineal gland. Biochem Biophys Res Commun 220:975–978
14. **Humphries A, Weller J, Klein D, Baler R, Carter DA** 2004 NGFI-B (Nurr77/Nr4a1) orphan nuclear receptor in rat pinealocytes: circadian expression involves an adrenergic-cyclic AMP mechanism. J Neurochem 91:946–955
15. **Coon SL, McCune SK, Sugden D, Klein DC** 1997 Regulation of pineal α1B-adrenergic receptor mRNA: day/night rhythm and β-adrenergic receptor/cyclic AMP control. Mol Pharmacol 51:551–557
16. **Price DM, Chik CL, Terriff D, Weller J, Humphries A, Carter DA, Klein DC, Ho AK** 2004 Mitogen-activated protein kinase phosphatase-1 (MKP-1): >100-fold nocturnal and norepinephrine-induced changes in the rat pineal gland. FEBS Lett 577:220–226
17. **Ganguly S, Coon SL, Klein DC** 2002 Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res 309:127–137
18. **Vanecek J, Sugden D, Weller J, Klein DC** 1985 Atypical synergistic α 1- and β-adrenergic regulation of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in rat pinealocytes. Endocrinology 116:2167–2173
19. **Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, Namboodiri MA, Coon SL, Hickman AB, Rollag M, Obsil T, Beauverger P, Ferry G, Boutin JA, Klein DC** 2001 Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14-3-3-binding switch in melatonin synthesis. Proc Natl Acad Sci USA [Erratum (2001) 98:14186] 98:8083–8088
20. **Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC** 1998 Melatonin production: proteasomal proteolysis in serotonin *N*-acetyltransferase regulation. Science 279:1358–1360
21. **Minneman KP, Iversen LL** 1976 Diurnal rhythm in rat pineal cyclic nucleotide phosphodiesterase activity. Nature 260:59–61
22. **Epplen JT, Kaltenhauser H, Engel W, Schmidtke J** 1982 Patterns of cyclic AMP phosphodiesterases in the rat pineal gland: sex differences in diurnal rhythmicity. Neuroendocrinology 34:46–54
23. **Houslay MD, Milligan G** 1997 Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem Sci 22:217–224
24. **Conti M, Jin SL** 1999 The molecular biology of cyclic nucleotide phosphodiesterases. Prog Nucleic Acid Res Mol Biol 63:1–38
25. **Swinnen JV, Joseph DR, Conti M** 1989 Molecular cloning of rat homologues of the Drosophila melanogaster dunce cAMP phosphodiesterase: evidence for a family of genes. Proc Natl Acad Sci USA 86:5325–5329
26. **Lobban M, Shakur Y, Beattie J, Houslay MD** 1994 Identification of two splice variant forms of type-IVB cyclic AMP phosphodiesterase, DPD (rPDE-IVB1) and PDE-4 (rPDE-IVB2) in brain: selective localization in membrane and cytosolic compartments and differential expression in various brain regions. Biochem J 304:399–406
27. **Monaco L, Vicini E, Conti M** 1994 Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. J Biol Chem 269:347–357
28. **Huston E, Lumb S, Russell A, Catterall C, Ross AH, Steele MR, Bolger GB, Perry MJ, Owens RJ, Houslay MD** 1997 Molecular cloning and transient expression in COS7 cells of a novel human PDE4B cAMP-specific phosphodiesterase, HSPDE4B3. Biochem J 328:549–558
29. **Farooqui SM, Al-Bagdadi F, Houslay MD, Bolger GB, Stout R, Specian RD, Cherry JA, Conti M, O'Donnell JM** 2001 Surgically induced cryptorchidism-related degenerative changes in spermatogonia are associated with loss of cyclic adenosine monophosphate-dependent phosphodiesterases type 4 in abdominal testes of rats. Biol Reprod 64:1583–1589
30. **Shepherd M, McSorley T, Olsen AE, Johnston LA, Thomson NC, Baillie GS, Houslay MD, Bolger GB** 2003 Molecular cloning and subcellular distribution of the novel PDE4B4 cAMP-specific phosphodiesterase isoform. Biochem J 370:429–438
31. **Lynch MJ, Hill EV, Houslay MD** 2006 Intracellular targeting of phosphodiesterase-4 underpins compartmentalized cAMP signaling. Curr Top Dev Biol 75:225–259
32. **Martin AC, Cooper DM** 2006 Layers of organization of cAMP microdomains in a simple cell. Biochem Soc Trans 34:480–483
33. **Houslay MD, Baillie GS** 2003 The role of ERK2 docking and phosphorylation

of PDE4 cAMP phosphodiesterase isoforms in mediating cross-talk between the cAMP and ERK signalling pathways. Biochem Soc Trans 31:1186–1190
34. **Houslay MD, Baillie GS** 2005 β-Arrestin-recruited phosphodiesterase-4 desensitizes the AKAP79/PKA-mediated switching of β2-adrenoceptor signalling to activation of ERK. Biochem Soc Trans 33:1333–1336
35. **Buda M, Klein DC** 1978 A suspension culture of pinealocytes: regulation of *N*-acetyltransferase activity. Endocrinology 103:1483–1493
36. **Ho AK, Chik CL** 1995 Phosphatase inhibitors potentiate adrenergic-stimulated cAMP and cGMP production in rat pinealocytes. Am J Physiol 268:E458–E466
37. **Ho AK, Ogiwara T, Chik CL** 1996 Thapsigargin modulates agonist-stimulated cyclic AMP responses through cytosolic calcium-dependent and -independent mechanisms in rat pinealocytes. Mol Pharmacol 49:1104–1112
38. **Moller M, Phansuwan-Pujito P, Morgan KC, Badiu C** 1997 Localization and diurnal expression of mRNA encoding the β1-adrenoceptor in the rat pineal gland: an in situ hybridization study. Cell Tissue Res 288:279–284
39. **Naro F, Sette C, Vicini E, De Arcangelis V, Grange M, Conti M, Lagarde M, Molinaro M, Adamo S, Nemoz G** 1999 Involvement of type 4 cAMP-phosphodiesterase in the myogenic differentiation of L6 cells. Mol Biol Cell 10:4355–4367
40. **Ho AK, Wiest R, Ogiwara T, Murdoch G, Chik CL** 1995 Potentiation of agonist-stimulated cyclic AMP accumulation by tyrosine kinase inhibitors in rat pinealocytes. J Neurochem 65:1597–1603
41. **Harper JF, Brooker G** 1975 Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′0 acetylation by acetic anhydride in aqueous solution. J Cyclic Nucleotide Res 1:207–218
42. **Chik CL, Ho AK** 1992 Ethanol reduces norepinephrine-stimulated melatonin synthesis in rat pinealocytes. J Neurochem 59:1280–1286
43. **Klein DC** 2006 Evolution of the vertebrate pineal gland: the AANAT hypothesis. Chronobiol Int 23:5–20
44. **D'Sa C, Tolbert LM, Conti M, Duman RS** 2002 Regulation of cAMP-specific phosphodiesterases type 4B and 4D (PDE4) splice variants by cAMP signaling in primary cortical neurons. J Neurochem 81:745–757
45. **Davis CW** 1984 Assessment of selective inhibition of rat cerebral cortical calcium-independent and calcium-dependent phosphodiesterases in crude extracts using deoxycyclic AMP and potassium ions. Biochim Biophys Acta 797:354–362
46. **Nemoz G, Prigent AF, Moueqqit M, Fougier S, Macovschi O, Pacheco H** 1985 Selective inhibition of one of the cyclic AMP phosphodiesterases from rat brain by the neurotropic compound rolipram. Biochem Pharmacol 34:2997–3000
47. **Parfitt A, Weller JL, Klein DC** 1976 β Adrenergic-blockers decrease adrenergically stimulated *N*-acetyltransferase activity in pineal glands in organ culture. Neuropharmacology 15:353–358
48. **Klein D, Weller JL** 1973 Adrenergic-adenosine 3′,5′-monophosphate regulation of serotonin *N*-acetyltransferase activity and the temporal relationship of serotonin *N*-acetyltransferase activity synthesis of ^{3}H-*N*-acetylserotonin and ^{3}H-melatonin in the cultured rat pineal gland. J Pharmacol Exp Ther 186:516–527
49. **Meyer RB, Jr., Miller JP** 1974 Analogs of cyclic AMP and cyclic GMP: general methods of synthesis and the relationship of structure to enzymic activity. Life Sci 14:1019–1040
50. **Baler R, Covington S, Klein DC** 1997 The rat arylalkylamine *N*-acetyltransferase gene promoter. cAMP activation via a cAMP-responsive element-CCAAT complex. J Biol Chem 272:6979–6985
51. **Binkley SA, Klein DC, Weller JL** 1973 Dark induced increase in pineal serotonin *N*-acetyltransferase activity: a refractory period. Experientia 29:1339–1340
52. **Roseboom PH, Klein DC** 1995 Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a β-adrenergic receptor/cyclic AMP mechanism. Mol Pharmacol 47:439–449
53. **Huang YY, Martin KC, Kandel ER** 2000 Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. J Neurosci 20:6317–6325
54. **Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ** 1997 Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron 19:1017–1030
55. **Appelbaum L, Toyama R, Dawid IB, Klein DC, Baler R, Gothilf Y** 2004 Zebrafish serotonin-*N*-acetyltransferase-2 gene regulation: pineal-restrictive downstream module contains a functional E-box and three photoreceptor conserved elements. Mol Endocrinol 18:1210–1221
56. **Appelbaum L, Vallone D, Anzulovich A, Ziv L, Tom M, Foulkes NS, Gothilf Y** 2006 Zebrafish arylalkylamine-*N*-acetyltransferase genes: targets for regulation of the circadian clock. J Mol Endocrinol 36:337–347
57. **Rath MF, Munoz E, Ganguly S, Morin F, Shi Q, Klein DC, Moller M** 2006 Expression of the Otx2 homeobox gene in the developing mammalian brain: embryonic and adult expression in the pineal gland. J Neurochem 97:556–566
58. **Chen W, Baler R** 2000 The rat arylalkylamine *N*-acetyltransferase E-box: differential use in a master vs. a slave oscillator. Brain Res Mol Brain Res 81:43–50
59. **Masumoto KH, Fujioka A, Nakahama K, Inouye ST, Shigeyoshi Y** 2003 Effect of phosphodiesterase type 4 on circadian clock gene Per1 transcription. Biochem Biophys Res Commun 306:781–785
60. **Prosser RA, Gillette MU** 1989 The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP. J Neurosci 9:1073–1081
61. **Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C** 2003 Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem 278:5493–5496
62. **Zatz M** 1992 Does the circadian pacemaker act through cyclic AMP to drive the melatonin rhythm in chick pineal cells? J Biol Rhythms 7:301–311
63. **Klein DC, Sugden D, Weller JL** 1983 Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal serotonin *N*-acetyltransferase. Proc Natl Acad Sci USA 80:599–603
64. **Sugden D, Weller JL, Klein DC, Kirk KL, Creveling CR** 1984 α-Adrenergic potentiation of β-adrenergic stimulation of rat pineal *N*-acetyltransferase. Studies using cirazoline and fluorine analogs of norepinephrine. Biochem Pharmacol 33:3947–3950
65. **Chik CL, Arnason TG, Dukewich WG, Price DM, Ranger A, Ho AK** 2007 Histone H3 phosphorylation in the rat pineal gland: adrenergic regulation and diurnal variation. Endocrinology 148:1465–1472

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